

Afferent and efferent pathways in the visual system of the freshwater snail *Planorbarius corneus*

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Abstract: Afferent and efferent neural elements of the retina and central ganglia in the freshwater snail *Planorbarius corneus* were labelled using retrograde transport of neurobiotin through the optic nerve. Axons of at least some photoreceptor cells become direct contributors to the optic nerve as no synaptic junctions could be detected. The processes enter the cerebral ganglion and form a dense bundle of thin afferent fibres, the so-called optical neuropil. Efferent neurons were revealed in all ganglia, except the buccal ones. Some of the ascending axons branch in the cerebral ganglia, cross the cerebro-cerebral commissure, reach the contralateral eye and form arborizations in the eye cup. Some efferent neurons send axons to different peripheral nerves as well: *n.n. intestinalis*, *pallialis dexter*, *pallialis sinister internus et externus*. Serotonin- and FMRF-amide-ergic fibres were revealed in the optic nerve. These fibres belong to those central neurons which send their axons to the ipsilateral eye only. They form abundant varicoses in the eye cup and nuclear layer of the retina, and possibly help to regulate retinal sensitivity to light.

Key words: Gastropoda; Nervous system; Eye; Retrograde transport; Serotonin; FMRF-amide

淡水蜗牛视觉系统的输入与输出通路

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摘要: 通过神经生物素在视神经上的逆行性传输对淡水蜗牛(*Planorbarius corneus*)视网膜及中央神经节的输入、输出神经元进行标记。由于没有发现突触联系,所以至少一部分光感受细胞的轴突可被视为直接参与形成视神经。这些神经元的轴突进入大脑神经节形成密集的细传入神经纤维束-视神经堆。传出神经元则存在于除颊部以外的所有神经节。一些上行轴突在大脑神经节处分叉,通过脑-脑联合,到达对侧眼并在眼杯处形成分枝。部分传出神经元的轴突也投射于不同的外周神经,如:*n.n. intestinalis*, *pallialis dexter*, *pallialis sinister internus et externus*。五羟色胺能纤维和 FMRF-酰胺能纤维均存在于视神经上,且这些纤维隶属于只投射在同侧眼的中央神经元。它们形成了位于眼杯处的丰富曲张结构及视网膜核心层,并且可能有助于调节视网膜对光的敏感性。

关键词: 腹足纲; 神经系统; 眼; 逆行性传输; 五羟色胺; FMRF-酰胺

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Retrograde transport of dyes like cobalt chloride and neurobiotin through the optic nerves of the freshwater snail *Lymnaea stagnalis* has shown the existence of neurons, which form connections between the two eyes and some internal organs (Zaitzeva, 1987; Zhukov & Tuchina, 2008). Moreover there are several central neurons, sending processes to both eyes (Tuchina et al, 2010). In order to investigate whether a connectivity

pattern like that of *L. stagnalis* was present in other pulmonate gastropods we carried out research on the freshwater snail *Planorbarius corneus*. This mollusc has well-developed camera-type eyes with a single lens (Paton & Kater, 1972), and its retinas contain many microvillar photoreceptors (Zhukov et al, 2002), whose central pathways are still not known.

The number of photoreceptor cells (Zhukov et al,

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2002; Bobkova et al, 2004) and the optical properties of the lens (Zhukov, 1993; Gál et al, 2004) in *P. corneus* suggest that eyes of this mollusc are to be involved in vision, although the corresponding behavioural reactions are not as evident as in *L. stagnalis* (Vakoljuk & Zhukov, 2000).

In the present work, we focused not only on the central visual pathways in *P. corneus*, but also on the efferent projections from brain/CNS to the eyes and the presence of some putative neurotransmitters, like 5-HT and FMRF-amide, in the retina as well. Serotonin- and FMRF-amide-ergic fibres are known to control the light sensitivity in eyes of the marine species, such as *Aplysia californica* (Corrent et al, 1978; Eskin & Maresh, 1982; Takahasi et al, 1989) and *Bulla gouldiana* (Jacklet et al, 1987); and it is also known that serotonin is present in the eyes of *L. stagnalis* as well, where it seemingly plays the similar role as in the marine snails (Zhukov et al, 2006).

1 Material and Methods

1.1 Animals

Adult specimens of *P. corneus* ($n=40$), collected from ponds around Bremen (Germany) during the period from autumn 2009 to spring 2010, were kept in aquaria at room temperature under 12L:12D light conditions and fed with cabbage or dandelion leaves twice a week. Before the experiments, the snails were immobilized and then dissected under a binocular microscope (Zeiss Stemi DV4, Göttingen, Germany).

1.2 Backfilling

The brain (or isolated eye with its optic nerve in the case of backfilling) was removed and transferred to a paraffin-filled Petri dish with phosphate-buffered saline (0.1 mol PBS, pH 7.4). All of the peripheral nerves except those we were interested in for backfilling like *n. opticus*, *n. pallialis sinister internus*, *externus*, *n. pallialis dexter* and *n. intestinalis* were cut at the side close to the corresponding ganglia to avoid penetration of the labelling solution. The cut ends of the nerves, which we were interested in, were placed in a tight vaseline pool, filled with distilled water, and exposed for approx. 1 min. The water was then replaced with labelling solution, 10% Neurobiotin (Sigma-Aldrich Inc., St.-Louis, MO). In some samples, Rhodamine B (Sigma-Aldrich Inc.) was added to the neurobiotin solution in order to visualize the ganglia under confocal microscopy. After 24–48 h (the duration of exposure depended on the

thickness of the nerve) of exposure to neurobiotin, the samples were fixed 3 h at room temperature or overnight at 4°C in 4% freshly made paraformaldehyde (PFA) in PBS, then washed at least 5 times in PBS (all further steps at 4°C, on a shaker) and finally cleaned with forceps.

Next, brain samples were put in blocking solution with 3% albumin fraction V (BSA) and 0.05% Triton X (TrX) in PBS for approx. 12 h; then washed several times with cold 0.1% BSA and 0.05% TrX in PBS; and exposed to antibody solution (Streptavidin-FITC or –Cy3: Sigma-Aldrich), diluted at 1:000 with 0.1% BSA and 0.05% TrX in PBS, for approx. 12 h. The samples then underwent another wash in cold PBS buffer, then they were cleaned in a graded series of ethanol (50, 70, 90, 2 × 100%, 10 min each), methylsalicylated (2–3 min) and finally embedded in Permount. Whole mount samples were observed under a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany), using LSM Image Browser and appropriate Zeiss software to make a series of confocal sections. Dichroic mirror HFT 488/543, emission filters LP 560 for Cy3 and BP 505-530 for FITC were used. The results obtained were processed using Adobe Photoshop CS (Adobe System Incorporated, San Jose, California, USA) and pen tablet Wacom Intuos3 (Wacom Co., Ltd, Saitama, Japan) for the preparation of schematic drawings. In some experiments simultaneous backfillings through both optic nerves were carried out (double labelling). In these cases one optic nerve was exposed to neurobiotin and another to lucifer yellow (LY) filter LP 475. Exposure to LY was performed the same way as to neurobiotin. For the experiments in which neurobiotin backfilling was combined with immunocytochemical staining to reveal putative neurotransmitters, the procedure for neurobiotin developing was the same as described above.

1.3 Immunocytochemistry

To identify neurotransmitters, the brain with optic nerves and eyes were removed, fixed in 10% freshly made PFA in PBS (pH 7.4) overnight at 4°C or 2–3 h at room temperature. When the immunocytochemistry procedure was combined with retrograde transport of neurobiotin through the optic nerve, backfilling with neurobiotin was carried out before the fixation. After the fixation all further steps were carried out on a shaker, at 4°C. The samples were washed in PBS, 5 times for 10 min, and incubated in 3% BSA overnight as described above for neurobiotin; then washed in 0.1% BSA with 0.05% TrX in PBS and exposed to primary antibody

solution (polyclonal rabbit anti-serotonin, Sigma Aldrich Inc., dilution 1:400, or rabbit anti-FMRF-amide, ImmunoStar, Mudson, WI, USA, dilution 1:400) for 2–3 days. Subsequently, the samples were washed again in 0.1% BSA with 0.05% TrX in PBS and put into secondary antibody solution (goat anti-rabbit antibodies-FITC conjugate, Sigma Aldrich Inc., dilution 1:1000) overnight. The whole mount samples were then washed in PBS buffer, cleaned in a graded series of ethanol (50, 70, 90, 2 × 100%, 10 min each), methylsalicilated (2–3 min), embedded in Permunt and finally observed under a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

For frozen sections, right after incubation in the secondary antibody, the samples were washed in PBS buffer, transferred to the molds, filled with freezing medium (Jung, Leica Microsystems, Nussloch, Germany), then freezed in liquid nitrogen for several minutes and cut on a Leica CM 1900 microtome (Leica Microsystems, Nussloch, Germany). Section thickness was 60 µm. The sections were then air-dried on microscope slides for 20–30 minutes, washed with PBS and dehydrated in a graded series of ethanol (50, 70, 90, 2 × 100%, 2 min each). In some samples prodipium iodide (Sigma Aldrich Inc.) was added prior to the dehydration with ethanol in order to identify the nuclei of the cells. The samples were then methylsalicilated (2

min), embedded in Permunt and finally observed under the confocal microscope.

2 Results

2.1 Backfilling through the optic nerve

Neurobiotin, transferred through the optic nerve of *P. corneus* to the central nervous system (CNS), labelled neuronal bodies in all ganglia except the buccal ones. The distribution of labelled neurons is shown in Tab.1. More neuronal bodies and their processes were identified in case of backfillings through the right optic nerve than the left one (Fig. 1). Stained bundles of fibres pass through the right and left sides of the CNS and it seems that they do not converge to each other, not even in the visceral ganglion (Fig. 2A). However, transit fibres form thin arborizations in all ganglia (Fig. 2B).

Fibres from the optic nerve formed two overlapping bundles of axons (neuropils) in the middle of the ipsilateral cerebral ganglion (Fig. 2C, D). One neuropil was small, but very dense, and located close to the input of the optic nerve, while another one, which was larger but more diffuse had a more central location. Double labelling with neurobiotin and lucifer yellow revealed that these two neuropils were formed by different fibres (Fig. 3A). Only one large neuropil, which was not dense, could be seen in the contralateral cerebral ganglion, and it is connected to the symmetrical neuropil from the

Tab. 1 Central neurons and fibres, revealed with backfilling through the optic nerve (left and right) in different ganglia of *Planorbarius corneus* (n=13)

	Left <i>n. opticus</i>	Right <i>n. opticus</i>	Neuronal bodies located regardless of the side of backfilling
RCG	Bundle of axons (optical neuropil)	3 medium sized neurons and 4 small ones on the dorsal side, 3 small ventral neurons, optical neuropil	—
LCG	Bundle of axons, projection to the contralateral <i>n. opticus</i>	2 medium sized and 3 small dorsal neurons, bundle of axons	—
RPIG	Transit fibres with arborizations in the neuropil of the ganglion	9 small dorsal neurons, 1 ventral neuron, transit fibres with arborizations, projection to the right pedal ganglion	—
LPIG	3 ventrally located neurons, transit fibres	5 dorsally located neurons, transit fibres with arborizations and projections to the left pedal ganglion	—
RPaG	Transit fibres with arborizations in the neuropil of the ganglion, projections to <i>n. pallialis dexter</i>	1 small ventrally located neuron, transit fibres and several axons to <i>n. pallialis dexter</i>	—
LPaG	2 dorsally and 1 ventrally located cell, transit fibres and projection to <i>n. pallialis sinister internus</i>	6 dorsally located neurons, 2 ventrally located, many transit fibres and thick arborizations, projection to <i>n. pallialis sinister internus</i>	2 neurons; one sending its axon to <i>n. pallialis sinister internus</i> , another to left pleural ganglion
RPeG	—	1 dorsally located neuron and 1 cell of statocyst	cell of statocyst
LPeG	1 dorsally located neuron and 1 cell of statocyst	1 ventrally located neuron and 1 cell of statocyst	cell of statocyst
VG	1 dorsal neuron, sending axon to <i>n. intestinalis</i>	2 dorsal neurons, 1 ventral neuron, projection to <i>n. intestinalis</i>	1 dorsal neuron, sending axon to <i>n. intestinalis</i>

Abbreviations: RCG, LCG – right and left cerebral ganglia, CCC – cerebro-cerebral commissure, RPIG, LPIG – right and left pleural ganglia, RPaG, LPaG – right and left parietal ganglia, VG – visceral ganglion, RPeG, LPeG – right and left pedal ganglia.

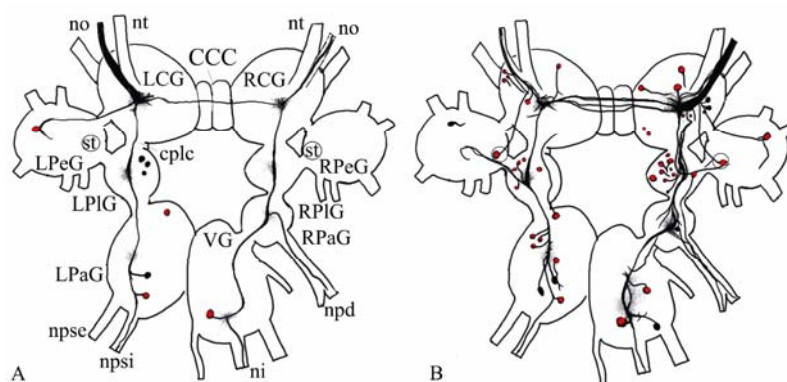


Fig. 1 Schematic drawings of the central visual pathways in *Planorbarius corneus*

CNS with neurons stained through the left (A) and right (B) *n. opticus*, interpedal commissure was cut. Abbreviations: RCG, LCG – right and left cerebral ganglia, RPIG, LPIG – right and left pleural ganglia, RPaG, LPaG – right and left parietal ganglia, VG – visceral ganglion, RPeG, LPeG – right and left pedal ganglia, CCC – cerebro-cerebral commissure, cplc – cerebro-pleural connective, st – statocyst, nt – *n. tentacularis*, no – *n. opticus*, npse, npsi – *n. pallialis sinister externus et internus*, pnd – *n. pallialis dexter*, ni – *n. intestinalis*. Dorsally located neurons are marked in red, ventral ones are in black.

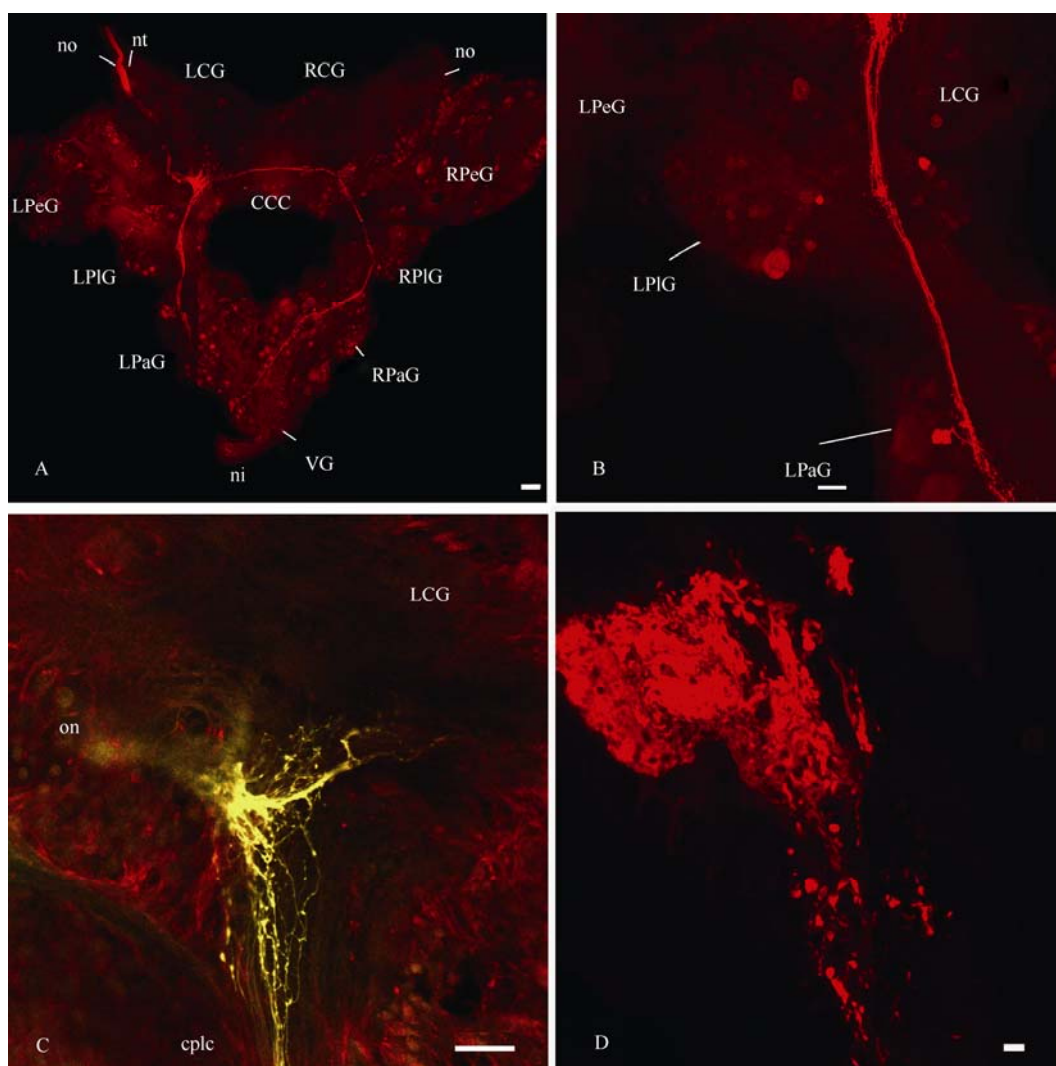


Fig. 2 Confocal micrographs of the central visual pathways in *Planorbarius corneus*

A) Circumoesophageal ring and different ganglia, backfilled with neurobiotin through the optic nerve in *P. corneus*; B) Thin arborizations stained in the left pleural and parietal ganglia through the left optic nerve; C,D) Dense optical neuropil in the left cerebral ganglion, whole mount sample and frozen section correspondingly. All ganglia except the pedal ones are shown from the dorsal side. On A, B and D neurobiotin is shown in red and on C in yellow. Abbreviations: see Fig. 1. Scale bar = 100 μ m on A, 50 μ m on B and C, 5 μ m on D. Section thickness is 60 μ m on D.

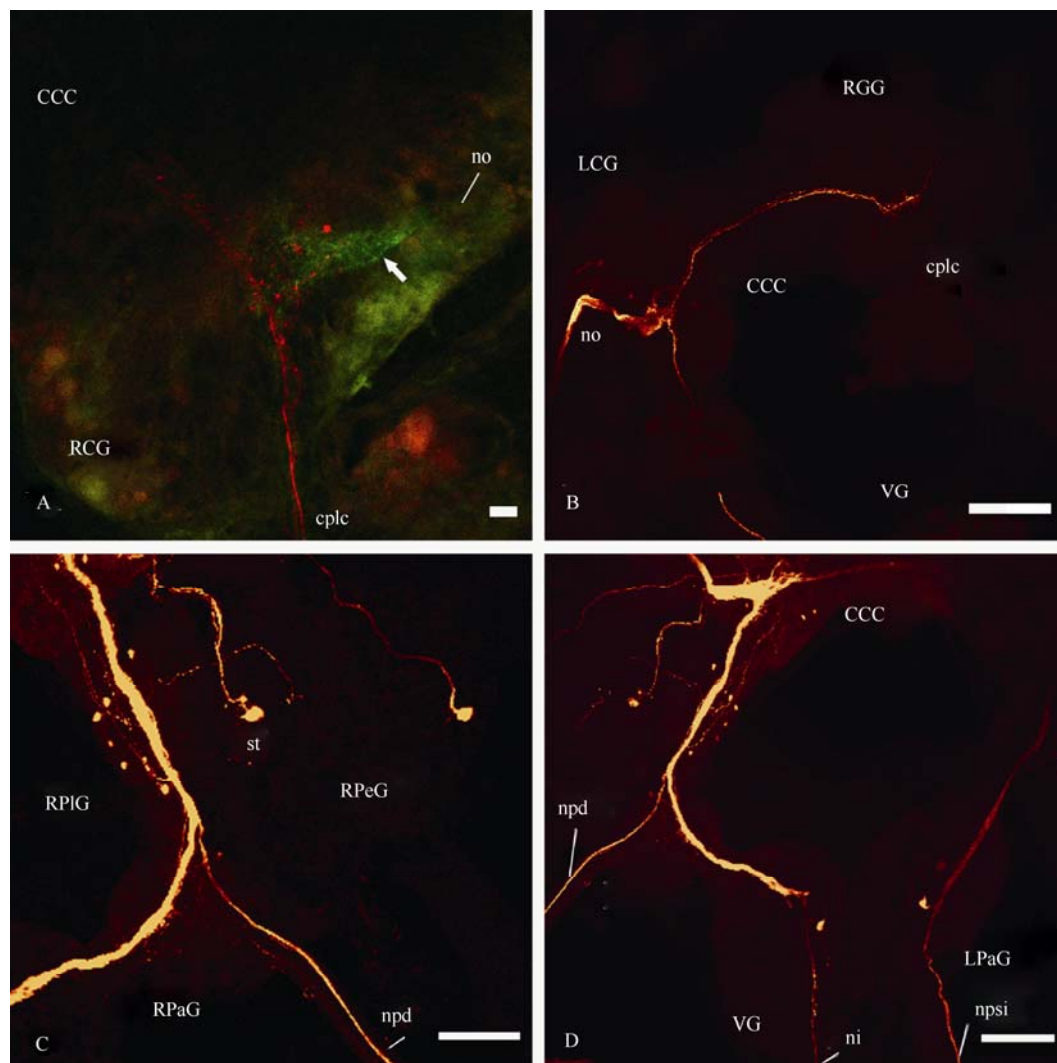


Fig. 3 Confocal micrographs of the central visual pathways in *Planorbarius corneus*

A) Right cerebral ganglion with two optical neuropils, revealed with double labelling with neurobiotin (backfilled through the left optic nerve) (red) and lucifer yellow (backfilled through the right optic nerve) (green), white arrow indicates the place where the right optic nerve enters the cerebral ganglion; B) Cerebral ganglia, filled with neurobiotin through the left optic nerve; C,D) Right pleural, pedal, parietal, visceral and left parietal ganglia, filled with neurobiotin through the right optic nerve, dorsal and ventral side correspondingly. On B, C and D neurobiotin is shown in yellow and on A in red. Abbreviations: see Fig.1. Scale bar = 20 μ m on A, 200 μ m on B, C and D.

ipsilateral ganglion via several axons in the cerebro-cerebral commissure (Fig. 3B). Two or three medium to large-sized neurons and several smaller ones were stained in the cerebral ganglia as well. The biggest number of labelled neurons was found on the dorsal surface of the pleural ganglia, especially the ipsilateral one. Axons of these cells joined in the bunch of transit fibres and went further to the cerebral and probably parietal ganglia. At least one axon from both sides of the circumoesophageal ring entered the pedal ganglion through the pleuro-pedal commissure. In the right parietal ganglion only a few transit fibres contributing (came from or went) to the *n. pallialis dexter* and only one neuron was stained (Fig. 3C).

In the left parietal ganglion, which was bigger than the right one (contrary to *L. stagnalis*), we revealed several quite large as well as some smaller neurons, whose axons jointly formed a bundle in the parietal neuropil. Axons of these cells ascended to the left cerebral ganglion where they joined the cerebral neuropil. Some axons passed along the *n. pallialis sinister internus* (Fig. 3D). In the unpaired visceral ganglion, three neurons were labelled; their fibres rise to the right cerebral ganglion. The ascending axon of one neuron, which is labelled on the ventral side of the visceral ganglion, forms arborization in the right cerebral ganglion and goes further through the cerebro-cerebral commissure (Fig. 4A). At least one axon from the

visceral neuropil joins the *n. intestinalis*. In each pedal ganglion one cell from the statocyst was stained through the *n. staticus*, and one neuron was revealed through the cerebro-pedal connective (Fig. 4B). Some neurons of the visceral, left parietal and probably other ganglia send their axons to both optic nerves and, thus, connect the eyes with each other. As a result of this, one or two

stained fibres are found in the contralateral optic nerve (Fig. 4C, D); they branch in the eye cup, but probably do not penetrate deep into the retina. Backfilling with neurobiotin through the optic nerve to the eye labelled photoreceptive cells, including microvillus-bearing parts (Fig. 10C, D), but no cell bodies were stained outside the retina.

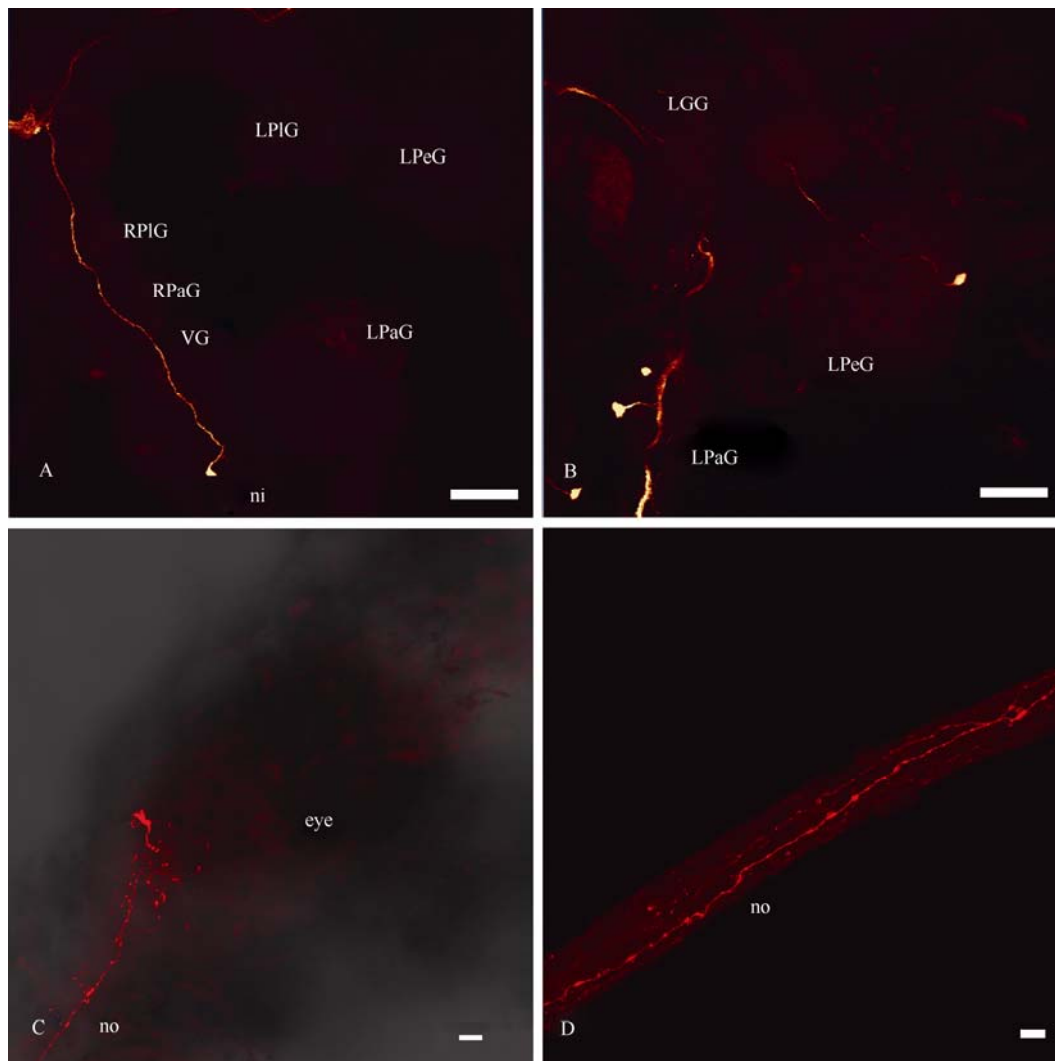


Fig. 4 Confocal micrographs of the central visual pathways in *Planorbarius corneus*

A,B) Neuron of the visceral ganglion, giving the projection to *n. intestinalis*, and neurons, stained in the left pedal and pleural ganglia correspondingly (photographs were taken from the ventral side); C,D) Projections to the contralateral eye and optic nerve correspondingly. On A and B neurobiotin is shown in yellow and on C and D – in red. Abbreviations: see Fig. 1. Scale bar = 200 μ m A and B, 20 μ m on C and D.

2.2 Backfilling with neurobiotin through *n. pallialis sinister internus*, *n. pallialis sinister externus*, *n. pallialis dexter* and *n. intestinalis*

These nerves were chosen, since they contain fibres, stained by backfilling with neurobiotin through the optic nerve. Many more neuronal bodies and axons were mapped through these nerves than through the optic one (Fig. 5). Staining revealed projections to the nerves of

cerebral, pedal, parietal and visceral ganglia. In case of backfilling through *n. pallialis dexter* and *n. pallialis sinister externus* some fibres in the *n. opticus* were also labelled (Tab. 2).

Fibres in the cerebro-cerebral commissure were labelled through all nerves (*n. pallialis sinister internus et externus*, *n. pallialis dexter* as well as *n. intestinalis*). However, their topographies were different. The upper

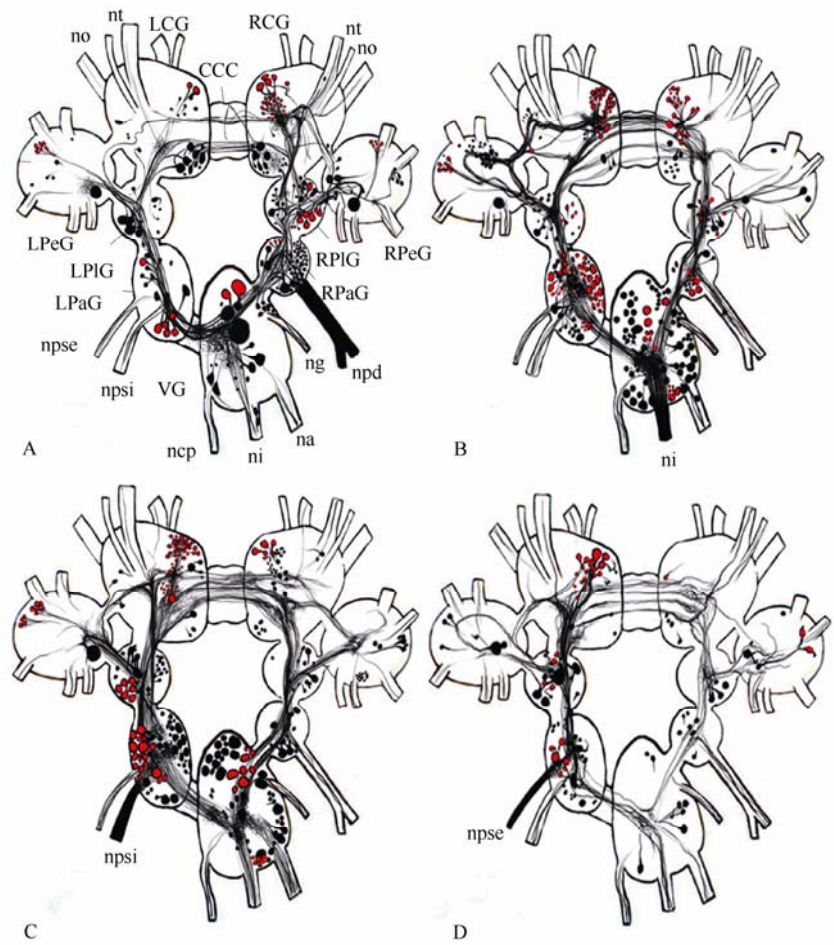


Fig. 5 Schematic drawings of the central pathways revealed through parietal and visceral nerves in *Planorbarius corneus* Backfilling through the *n. pallialis sinister externus* (A), *n. intestinalis* (B), *n. pallialis sinister internus* (C), *n. pallialis sinister externus* (D). Dorsally located neurons are marked in colour, ventrally located ones in black. Abbreviations: see Fig. 1.

Tab. 2 Projections of *n. pallialis sinister externus* et *internus*, *n. intestinalis* and *n. pallialis sinister externus* into different nerves

Nerves, which were subjected to backfilling	Projections labelled in different nerves										
	<i>n.pallialis sinister externus</i>	<i>n.pallialis sinister internus</i>	<i>n.intestinalis</i>	<i>n.cutaneus pallialis</i>	<i>n.analis</i>	<i>n.genitalis</i>	<i>n.pallialis dexter</i>	Right <i>n.tentacularis inferior</i>	Left <i>n.tentacularis inferior</i>	Right <i>n.opticus</i>	Left <i>n.opticus</i>
<i>n. pallialis sinister externus</i>		+	+	+	+	+	+	+	+	+	—
<i>n. pallialis sinister internus</i>	+		+	+	+	+	+	+	+	—	—
<i>n. intestinalis</i>	+	+		+	+	+	+	+	+	—	—
<i>n. pallialis dexter</i>	+	+	+	+	+	+		+	+	+	—

“+” means presence of stained fibres. “–” means absence of stained fibres.

bundle of fibres (marked with arrows in Figs. 6C, 7A, 9A) connects symmetrical clusters of neurons in the mesocerebra of the cerebral ganglia, while the lower bundle (Fig.6A) connects groups of neurons in the caudal regions of the ganglia. Most fibres in the cerebro-cerebral commissure were stained through the *n.*

intestinalis and *n. pallialis sinister internus*.

Backfilling through the *n. pallialis sinister externus* revealed four thin bundles of axons, and staining through the *n. pallialis sinister* turned up two well-defined bundles. Labelled fibres form a triangle-like structure in the cerebral neuropil (marked with arrows in Figs. 6B, 8A,

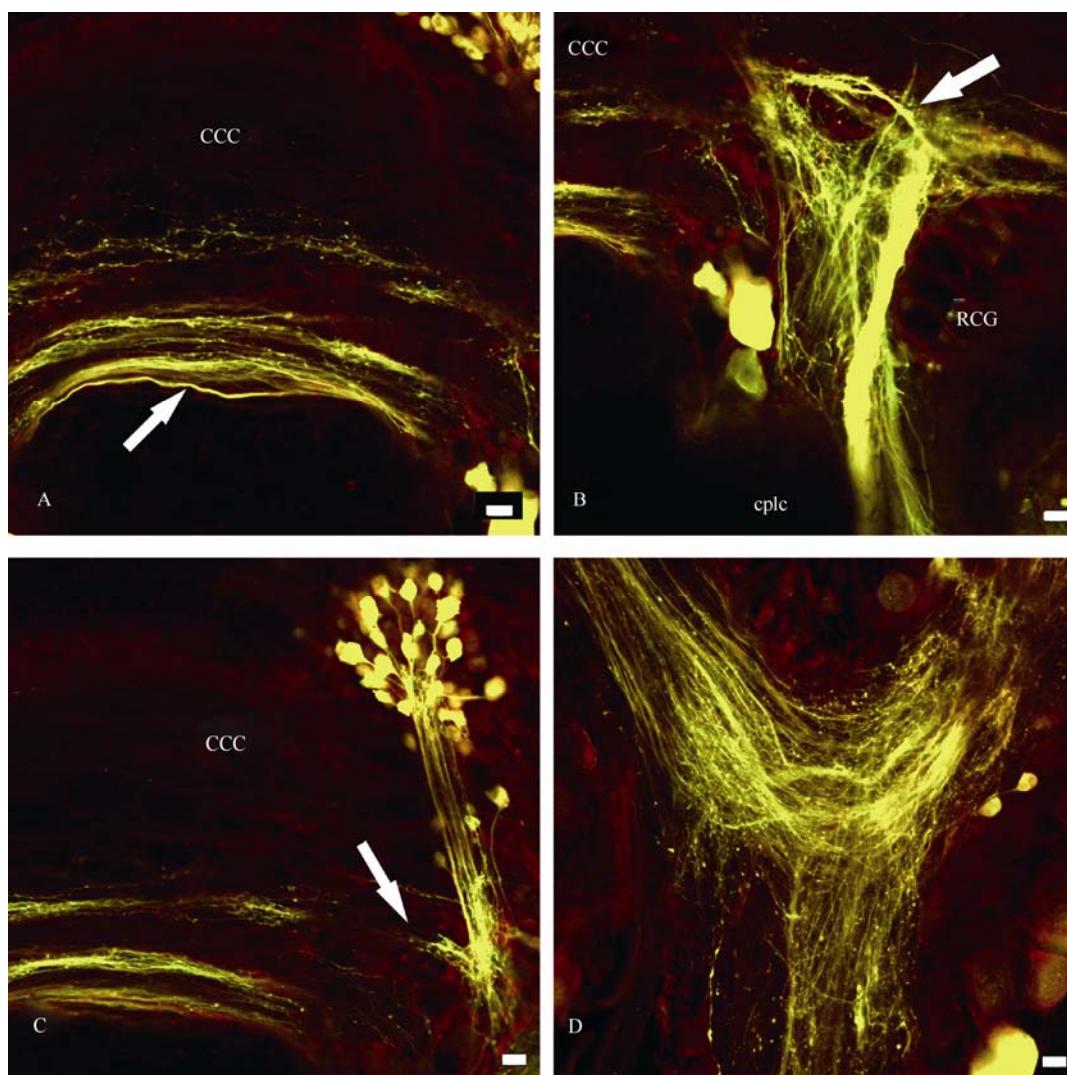


Fig. 6 Confocal micrographs of the central pathways of *n. pallialis dexter* in *Planorbarius corneus*

A) Labelled fibres in the cerebro-cerebral commissure; white arrow shows a lower bundle of stained fibres, which connect groups of neurons in the caudal regions of the ganglia; B) Axonal plexus in the right cerebral ganglion; white arrow marks triangle-like structure in the cerebral neuropil; C) Cluster of small cells in the mesocerebrum of right cerebral ganglion; white arrow indicates fibres in cerebro-cerebral commissure, which connects symmetrical clusters of neurons in the mesocerebra of the cerebral ganglia; D) Neuropil in the visceral ganglion. Abbreviations: see Fig. 1. Scale bar = 20 μ m.

B), from which several processes lead into the *n. tentacularis* and *n. opticus*.

From the cerebral ganglia, transit fibres went to pedal, pleural, and parietal ganglia on each side of the CNS and finally join in the visceral ganglion (Fig. 6D, 7D, 8C, 9D). Thus, the projections form a closed ring and send processes into most of the peripheral nerves, including pedal, parietal and visceral nerves. It is to be noted that transit fibres form two well-defined bundles of axons (Fig. 6B, 9B).

The same groups of neurons were labelled through various nerves in the pedal ganglia (Fig. 7B, 8D, 9C).

Primarily, all these are two groups of small neurons, which seem to have a symmetrical location and voluminous cells near the cerebro-pleural connective. Groups of neurons send their processes mostly to the corresponding pleural ganglion; large cells send processes to some pedal nerves as well as to the corresponding, i.e., ipsilateral, cerebral and pleural ganglia.

Numerous transit fibres and neurons were mapped in both pleural ganglia (Fig. 8B, 9B), in case of *n. pallialis dexter* especially in the right pleural ganglion, but in the case of *n. pallialis sinister externus et internus*

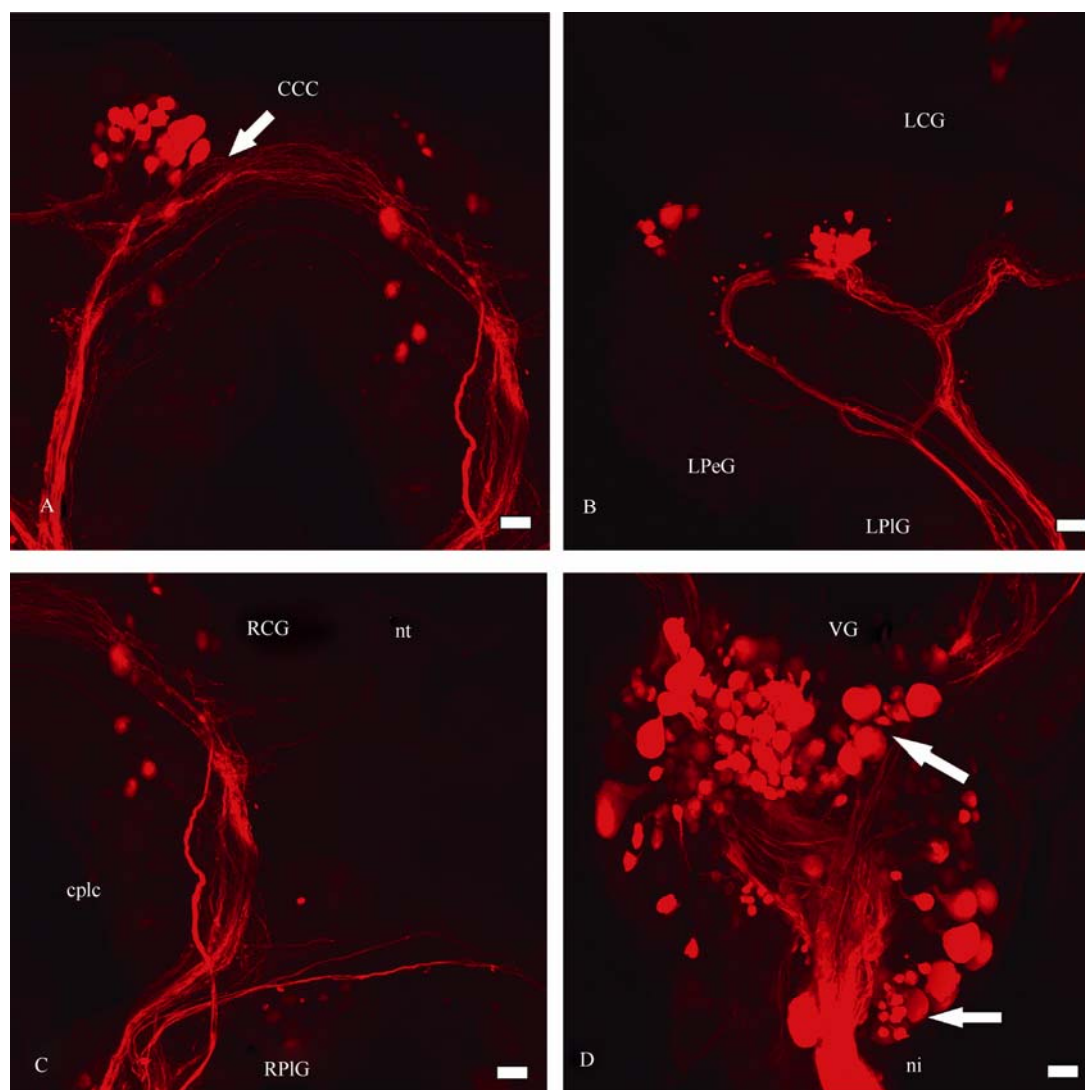


Fig. 7 Confocal micrographs of the central pathways of *n. intestinalis* in *Planorbarius corneus*

A) Labelled neurons and fibres in the cerebro-cerebral commissure and cerebral ganglia; white arrow marks fibres in the cerebro-cerebral commissure, which connects symmetrical clusters of neurons in the mesocerebra; B) Groups of labelled cells in the left pedal ganglion, C) Transit fibres in the right cerebral and pleural ganglia; D) Neurons in the visceral ganglion; white arrows indicate two identical groups of dorsally located neurons were stained through the *n. intestinalis* and *n. pallialis sinister internus*. Abbreviations: see Fig.1. Scale bar = 50 μ m on A, B and C, 20 μ m on D.

in the left one. Groups of cells, which seem to have a symmetrical location in the right and left pleural ganglia, were stained through the *n. pallialis dexter*, and some cells of this group were likely stained through other nerves: one cell in the left pleural ganglion and two cells in the right one in case of backfillings through the intestinal nerve, and 2-3 cells in each pleural ganglion in case of the staining of left pallial nerves.

As for the parietal ganglia, the largest amount of neurons and fibres in the left parietal ganglion was labelled through the *n. pallialis sinister internus*. Firstly, there is a large group of dorsally located cells close to the site where the pallial nerves enter the ganglion; then

there are several ventrally located cells in the caudal region of the ganglion and one more group of neurons, which is located in the proximal region of the ganglion. The latter group can be defined in the case of backfilling through the *n. intestinalis* as well. Neuronal structures in the right parietal ganglion were stained more clearly after backfilling through the *n. pallialis dexter*. However, the topography of the transit fibres and thin axons can be distinguished more easily in case of backfillings through other nerves.

Numerous neurons and fibres were labelled in the unpaired visceral ganglion (Fig. 6D, 7D, 8C, 9D). Two identical groups of dorsally located neurons were stained

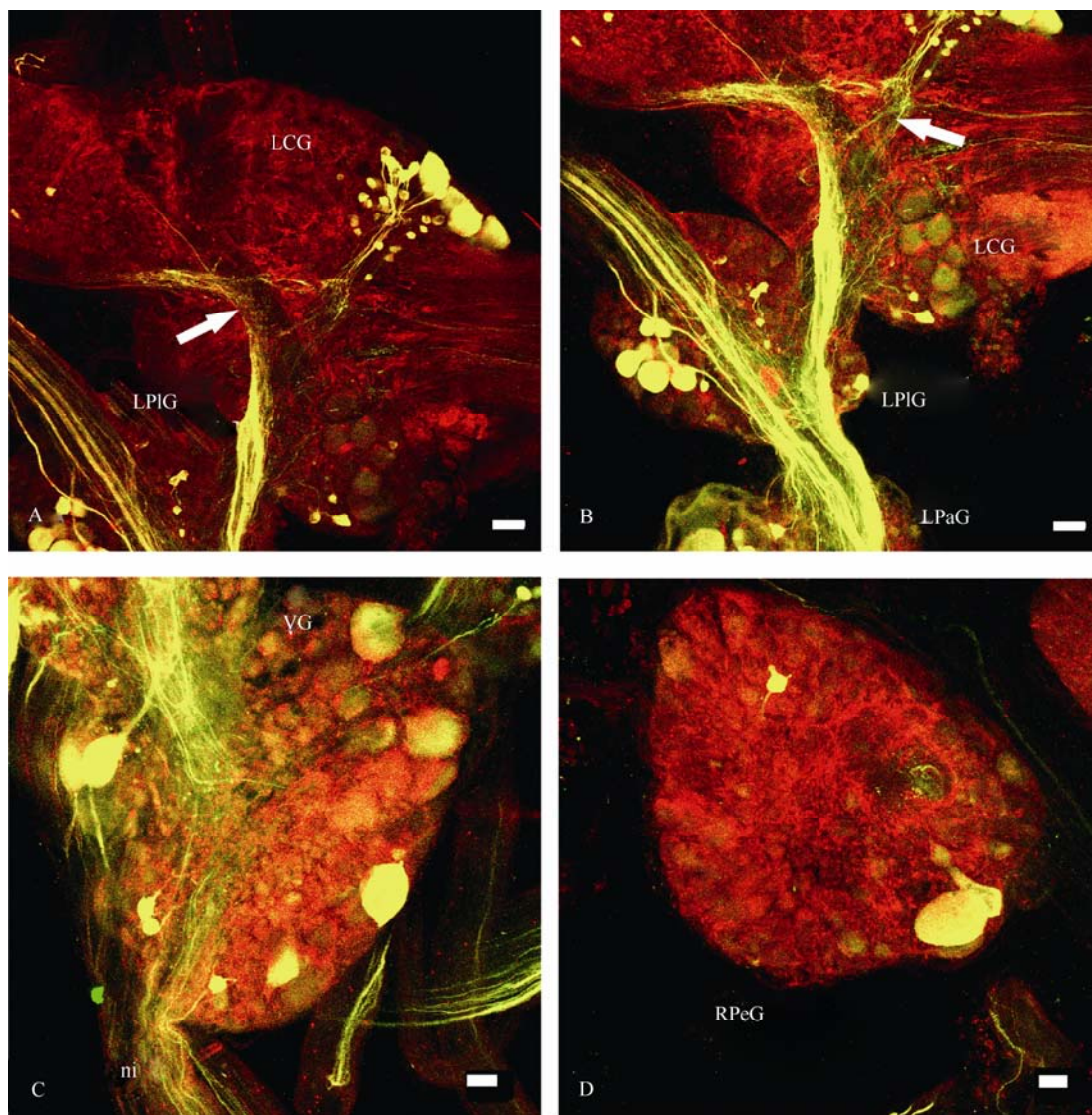


Fig. 8 Confocal micrographs of the central pathways of *n. pallialis sinister internus* in *Planorbarius corneus*

A) cluster of cells in the mesocerebrum of left cerebral ganglion; B) transit fibres and labelled neurons in the left cerebral and pleural ganglia; white arrows on A and B mark triangle-like structure in the cerebral neuropil; C) neurons and axons in the visceral ganglion; D) labelled neurons in the right pedal ganglion. Abbreviations: see Fig.1. Scale bar = 50 μ m.

through the *n. intestinalis* and *n. pallialis sinister internus* (marked with arrows on Fig.7D and 9D). Projections enter all visceral nerves, but they become more apparent through retrograde transport along the *n. pallialis sinister internus* and *n. intestinalis*.

2.3 Immunocytochemistry

Serotonin- and FMRF-amide-ergic fibres were revealed immunocytochemically in the CNS, optic nerve and the eye of *P. corneus* (Tab.3). Several 5HT-ergic fibres with varicoses were mapped in the optic nerve, and branches with many varicoses were abundant in the eye cup (Fig. 10). The stained branches were seen mostly at the base of the nuclear layer of the retina (Fig. 10C). It is difficult to follow the thin serotonergic fibres

(labelled in the optic nerve) down to the CNS. They end somewhere in the mid region of the ipsilateral cerebral ganglion. Retrograde transport of neurobiotin through the optic nerve together with an application of serotonin antibodies allowed us to compare central visual pathways with 5HT-ergic neurons and fibres (Fig. 11). The two do not seem to coincide.

As for FMRF-amide, there are many thin FMRF-amide-ergic fibres with varicoses in the sheath of the CNS and the optic nerve in particular (Fig.12A, B). These fibres enter the eye capsule and then form a varicosed branch there. FMRF-ergic fibres differ from those stained with neurobiotin through the contralateral optic nerve (Fig.12B), although their central visual

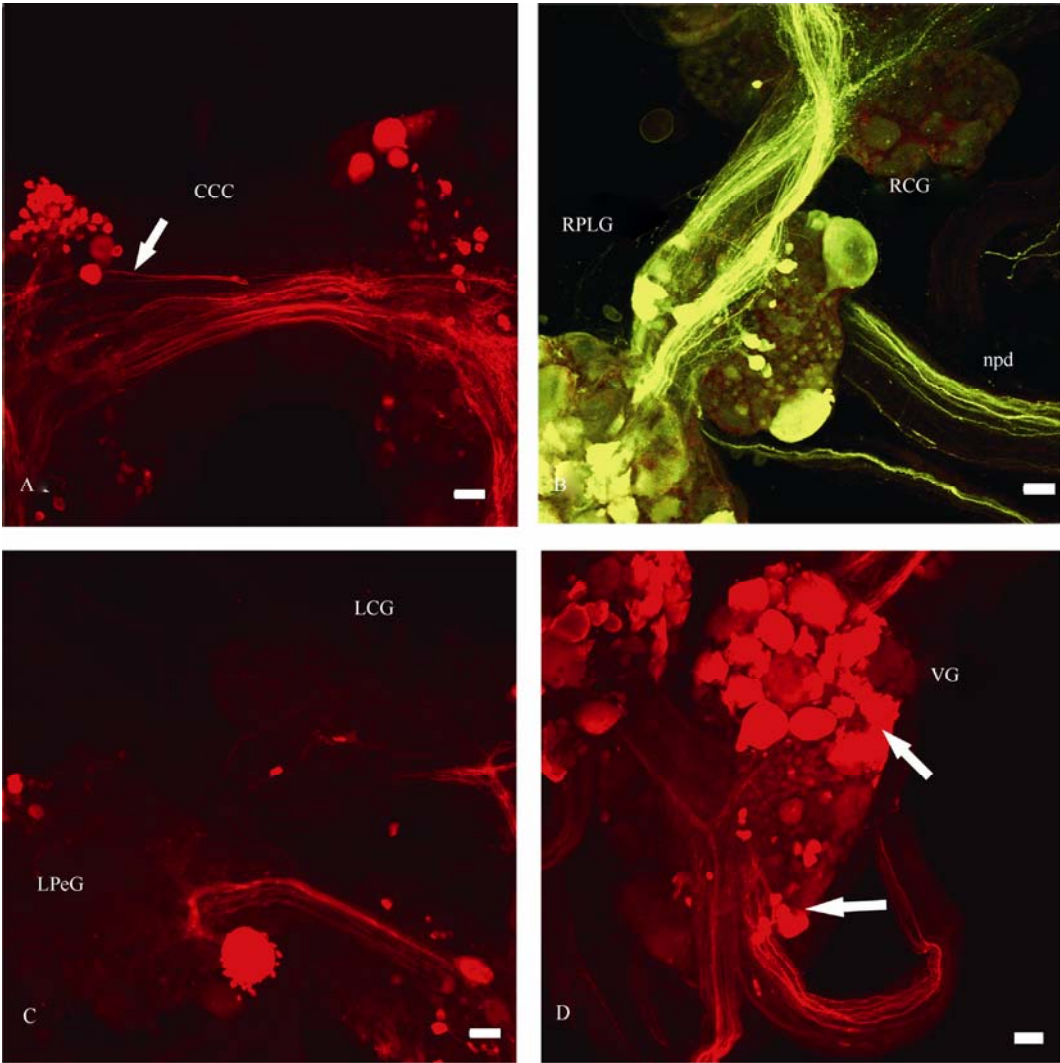


Fig. 9 Confocal micrographs of the central pathways of *n. pallialis sinister externus* in *Planorbarius corneus*
A) Labelled neurons and fibres in the cerebro-cerebral commissure and cerebral ganglia, white arrow indicates fibres in the cerebro-cerebral commissure, which connects symmetrical clusters of neurons in the mesocerebra; B) Transit fibres and labelled cells in the right pleural ganglion; C) Neurons and fibres in the left pedal ganglion; D) Neurons and projections in the visceral ganglion, white arrows mark two identical groups of dorsally located neurons stained through the *n. intestinalis* and *n. pallialis sinister internus*. On A, C and D neurobiotin is shown in red and on B in yellow. Abbreviations: see Fig.1. Scale bar = 50 μ m.

Tab. 3 Localization of the serotonin- and FMRF-amide-ergic cells and fibres in the eye optic nerve and central neural system of *Planorbarius corneus*

	5HT	FMRF-amide
Eye	branches and varicoses	branches
<i>n. opticus</i>	3–4 fibres with varicoses	1–2 fibres in the sheath
CNS:	many cells and fibres,	many cells and fibres in ganglia,
Cerebral ganglia	2 big symmetrically located neurons in the mesocerebrums, 2 symmetrical groups of neurons near the cerebro-cerebral commissure in each ganglion, and several small single cells	
Cerebro-cerebral commissure	2 bundles of fibres	several fibres, which do not form bundles
Pedal ganglia	many neurons, at least 1 big cell near the pleura-pedal connective in each ganglion	many neurons and fibres
Pleural and parietal ganglia	2 transit fibres, several small cells in the middle of the pleural ganglia, group of small neurons in the left parietal ganglion, close to the parieto-visceral connective	transit fibres in pleural and parietal ganglia, group of cells in the left parietal ganglion
Visceral ganglion	transit fibres, several small cells, 1 middle-sized neuron in the middle of the ganglion	transit fibres and several cells

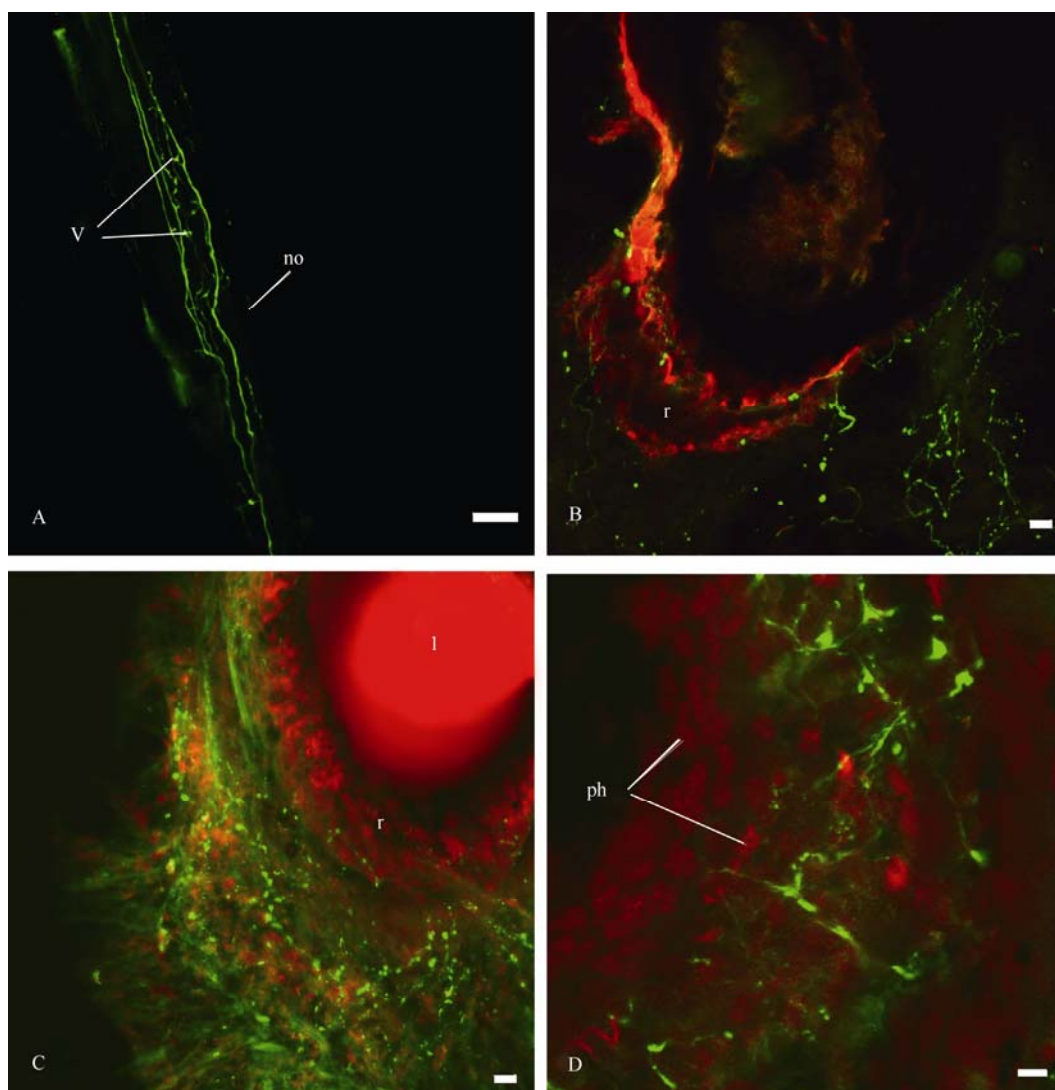


Fig. 10 Distribution of 5HT-fibres and varicoses in the optic nerve (A) and the eye (B, C and D) of *Planorbarius corneus*. Serotonin is shown in green colour, red is propidium iodide. Abbreviations: no – *n. opticus*, l – lens, r – retina, ph – nuclei of photoreceptors, v – varicoses on 5HT-fibres. Scale bar = 20 μ m on A, 10 μ m on B and C, 5 μ m on D.

pathways can also be revealed in all of the ganglia (Fig.12C, D). Only fibres, but no 5HT- or FMRF-amid-immunoreactive cell bodies within the eye cup were revealed by staining.

3 Discussion

3.1 Earlier findings

In earlier investigations on the visual pathways in gastropods, cobalt compounds were generally used (Jacklet et al, 1982; Zaitseva et al, 1982; Ovchinnikov, 1986). These tracers (as well as lucifer yellow and rhodamine-dextran) label central neurons and fibres only in the cerebral ganglia possibly because of the small diameter of the fibres in the optic nerve. For example,

retrograde transport of rhodamine-dextran through the pedal nerves in *L. stagnalis* results in the visualization of numerous neurons and fibres, stained throughout the oesophageal ring (Kononenko, Zhukov, 2005), while backfilling through the optic nerve allowed us to reveal only the optic neuropil (Zhukov & Tuchina, 2006). To reveal more details, neuronal processes can be labelled using horseradish peroxidase (HRP) (Olson & Jacklet, 1985; Lacroix et al, 1991), but the best results for *L. stagnalis* and *P. corneus* were achieved with neurobiotin (Zhukov & Tuchina, 2008). Double labelling with neurobiotin and lucifer yellow allowed us to distinguish (a) afferent fibres, which stem from the ipsilateral optic nerve and form the optical neuropil in the cerebral

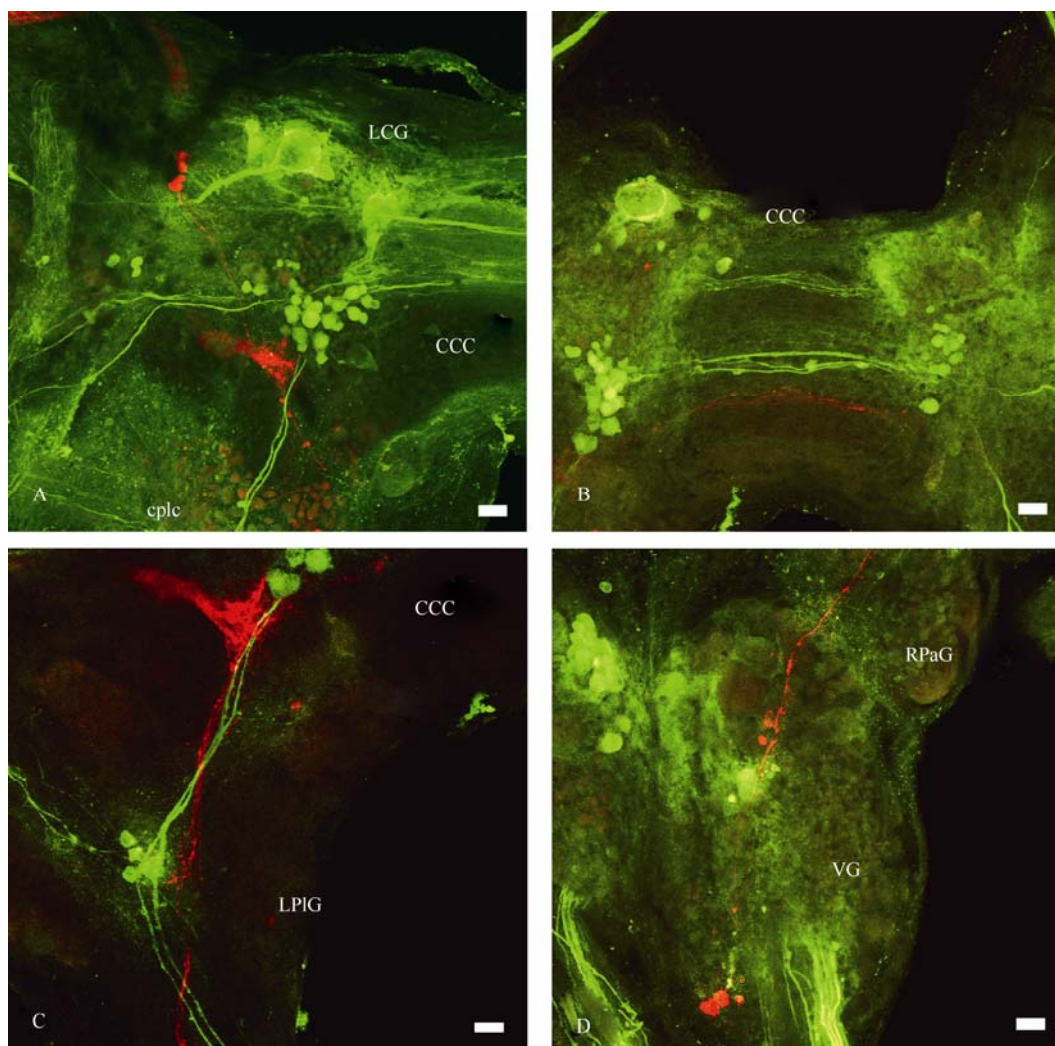


Fig. 11 Distribution of the central visual pathways (red) and 5HT-ergic neurons and fibres (green) in the CNS of *Planorbarius corneus*. A) The left cerebral ganglion, backfilling from the left optic nerve; B) Fibres in the cerebro-cerebral commissure; C) Distribution of projections in the left cerebral and pleural ganglia; D) Neurons and processes labelled in the visceral ganglion. Abbreviations: see Fig.1. Scale bar = 50 μ m.

ganglion, and (b) efferent fibres, which come from the contralateral nerve which seemingly belong to the neurons of visceral and left parietal ganglia. Efferent fibres interact with afferent ones by forming arborizations and varicoses in the optical neuropil.

The optical neuropil in *P. corneus*, formed by afferent fibers of the optic nerve, is a dense bundle of thin axons, i.e., processes of photoreceptor cells, which seems to be a very characteristic structure of gastropods. Its presence in *L. stagnalis* and *Helix lucorum* has already been verified by Zhukov & Tuchina (2008) and Ovchinnikov (1986), respectively. In the land snail *Helix* sp. axons of photoreceptors synapse with secondary neurons in an enlargement of the optic nerve, and, thus, the optical neuropil, labelled in the ipsilateral cerebral

ganglion in this snail, is formed by processes of secondary cells quite unlike the situation of the freshwater snails *L. stagnalis* and *P. corneus*.

3.2 Pathways of photoreceptor neurons

Although some neurons were discovered in the retina of *L. stagnalis* (Bobkova, 1998), we still possess little information about their function (since the presence of a circadian oscillator in the eye of *L. stagnalis* is still under investigation). Most of the photoreceptors send their axons directly to the optic nerve, allowing us to use backfilling as a method of identification for the central visual pathways (Zhukov, 2007). Based on the results from the present work obtained through anterograde transport, we suggest that the optical neuropil in *P. corneus* is also formed mainly by processes of the

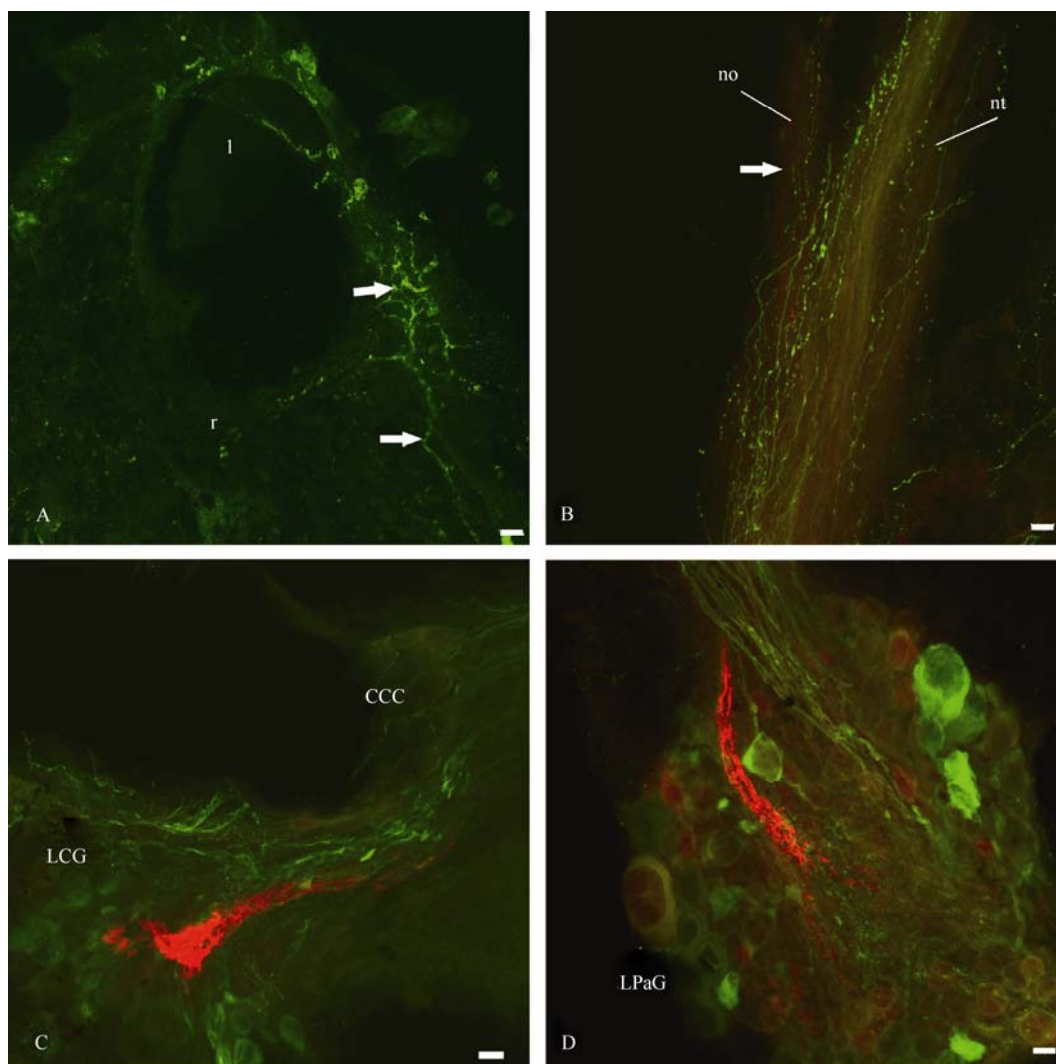


Fig. 12 Distribution of the central visual pathways (red) and FMRF-amide-ergic neurons and fibres (green) in the eye (A), optic nerve (B) and CNS (C and D) of *Planorbarius corneus*.

A) FMRF-amide-ergic fibres near the eye-cup; B) FMRF-amide-ergic fibres in the tentacular and optic nerves and visual projections from the contralateral optic nerve (marked with arrow); C) Pathways stained by neurobiotin in the left cerebral ganglion through the ipsilateral optic nerve; D) Neurons and processes labelled in the left parietal ganglion through the left optic nerve. Abbreviations: see Fig. 1 and 10. Scale bar = 20 μ m.

photoreceptive cells.

The number of cells in the retina of some of the snails mentioned above is quite high. For example, it is about 2,500 in *L. stagnalis* (Gal et al, 2004), around 5,000-7,000 in *A. californica* (Jacklet, 1969; 1973) and approximately 1,000 in *B. gouldiana* (Jacklet, Colquhoun, 1983). Based on the density of the apices of the photoreceptors in the retina of *P. corneus*, the number of photoreceptor cells in the eye of this snail ranges from several hundreds to more than two thousand (Zhukov et al, 2002; Gál et al, 2004), which allows us to estimate that there have to be numerous synaptic contacts in the optical neuropil.

As for the marine species *Aplysia californica* and

Bula gouldiana, the localization of the afferent visual pathways is still under investigation. In addition to the photoreceptor cells in the retinas of these two species, there are also neurons of a circadian oscillator (Block & McMahon, 1984; Block et al, 1984; Jacklet, 1984). Axons of these neurons as well as axons of the photoreceptive cells form the optic nerve. Olson & Jacklet (1985) and other authors (Lacroix et al, 1991) were trying to separate these two kinds of fibres and mark them with HRP, precursors of serotonin or 3 H-leucine, but a clear answer about the exact localization of the afferent fibres has not been achieved. Most likely the plexus of fibres, which was stained in the lateral region of the cerebral ganglion in the aforementioned two

species, is formed at least partially by afferent optical fibres, comparable to the optical neuropil of freshwater snails. Labelling of the CNS of *Aplysia* through the optic nerve revealed cell bodies and fibres in almost all of the ganglia, and these stained fibres form arborizations in the neuropils of the ganglia. Although authors like Olson & Jacklet suggested that these projections belonged to the circadian oscillator system and their widespread distribution in the brain of *Aplysia* was due to the need to adjust a variety of functions to the light cycle, we think that the visual pathways (i.e., processes of photoreceptors) may contribute as well.

Staining of the CNS through the optic nerve in *P. corneus* revealed bodies of neurons that send their axons to the optic nerve and are likely to represent efferents of the eye. Similar neurons were labelled in the cerebral ganglia of *Aplysia* (Olson & Jacklet, 1985) and *Helix* (Ovchinnikov, 1986). In *Aplysia* these neurons form two groups, which innervate the eye through the main and additional optic nerves. The overall distribution of the labelled neurons in the CNS of *P. corneus* is similar to that described in *L. stagnalis* (Zhukov & Tuchina, 2008; Tuchina et al, 2010). First of all, the labelled neurons are located in all of the ganglia except the buccal ones. The most remarkable cells were identified in the parietal and visceral ganglia, since their axons reach the retinas of both eyes. One of the presumed functions of these cells is to compare information on light conditions reaching the right and left eyes, and thus to identify the direction of the light source. *P. corneus*, just like *L. stagnalis*, exhibits a positive phototaxis (Zhukov et al, 2002).

The eyes of *B. gouldiana* are functionally connected, since the electrical activity of the retinal cells in one eye can be registered in the contralateral optic nerve, and this feature is probably important for the synchronization of both circadian oscillators (Roberts & Block, 1985). But the morphological basis for these connections is still missing: the central pathways, which were labelled with HRP, can be followed only through the cerebro-cerebral commissure towards the contralateral eye (Lacroix et al, 1991). Since HRP-stained fibres are also found in the pleural ganglion and in the pleura-parietal connective, there are probably some neurons, which are able to connect both eyes as in *L. stagnalis* and *P. corneus*. In *H. crassicornis*, whose retina is rather simple, the eyes are connected through the neurons of the optic ganglion, i.e. secondary neurons (Alkon, 1973). Interestingly, direct connections between the eyes of *Aplysia*, which like

those of *Bulla gouldiana* have circadian oscillators, in accordance with Olson & Jacklet (1985), were not revealed. However, in the related snail *Bursatella leachii pleii* circadian oscillators have been reported to be highly synchronized (Roberts et al, 1987).

3.3 Neuronal projections

Another remarkable feature in the topography of the central visual pathways in *P. corneus* is projections to the pedal ganglia and particularly to the statocysts, something that had also been shown in *L. stagnalis* (Tuchina et al, 2010), these connections probably provide the basis for phototaxis (Vakoljuk & Zhukov, 2000) and visuo-vestibular associative learning (Sakakibara et al, 1998; Sakakibara, 2006). It is to be noted that behavioural patterns in *P. corneus* are poorly studied, but we suggest that interactions between visual and vestibular information processing could be important for the orientation of these snails towards the light, i.e., the surface of their aquatic habitat, for breathing.

As in *L. stagnalis*, some of the labelled central neurons in *P. corneus* send their processes along peripheral nerves. By using retrograde transport of neurobiotin through these nerves we were able to reveal projections to the optic nerve only for *n. pallialis dexter internus*, but most likely such projections also occur with regard to the *n. pallialis dexter externus* and *n. intestinalis* also. The neurons, which connect different nerves, are very typical for the CNS of *L. stagnalis*, where they form mononeuronal reflex arcs (Zaitseva, 1982; 1987). Such neurons can be expected to be present in the neural system of *Bulla* and *Aplysia*, since after the backfilling of the optic nerve some stained fibres are found in peripheral nerves. In *Aplysia* such fibres were presented on the right and left sides of the *n. tentacularis* and *n. frontolabialis superior* (Olson & Jacklet, 1985). We conclude that mononeuronal connections between different peripheral parts of the body are rather typical in gastropods.

3.4 Efferent innervation

Efferent innervations of the retina are well known from arthropods, but they are present in cephalopods as well. In octopus the bodies of the efferent dopaminergic neurons are located in the optical lobe; they are electrically coupled with photoreceptors and are able to change the responses of the photoreceptor cells, as well as control the screening pigment migration (Suzuki, Tasaki, 1983; Gleadall et al, 1993).

Observations on efferent innervations of the retina

in *Chelicerata* involved: spiders (Yamashita, 1990; Uehara et al, 1993), scorpions (Fleissner, Schliwa, 1978) and the horseshoe crab *Limulus polyphemus* (Calman, Battelle, 1991). In the scorpion *Androctonus australis* there is a stem of 10-20 central neurons, which is located near the oesophagus, each neuron of this group sending processes to both eyes (Fleissner & Fleissner, 2002). The electrical activity of these cells depends on the phase of the circadian cycle. Depending on the timing of the cycle a different amount of octopamine is secreted in order to modulate the screening pigment migration and, thus, the state of light adaptation of the eye (Fleissner & Fleissner, 1978, 2002). In the spider *Argiope sp.* efferent innervations of the retina are achieved by neurons of the protocerebrum, which modulate the light sensitivity of the photoreceptors, and probably exert an effect on synthesis and degradation of photoreceptor membranes (Yamashita & Tateda, 1981). The electrical activity of these protocerebrum neurons changes according to the illumination of the retina (Yamashita, 1990). Moreover, they themselves are light-sensitive (Yamashita & Tateda, 1983; Yamashita, 2002).

The efferent innervations of the lateral eye retina in *Limulus* have been studied in great details. Some of the efferent neurons, which are located in the cheliceral ganglia, innervate both eyes (Calman & Battelle, 1991). Other cells, located in the protocerebrum in separate groups, are connected to each other through the commissure and probably affect both eyes as well (Saito et al, 2004). The efferent fibres of the retina contain octopamine (Battelle et al, 1982), which changes the light sensitivity of ommatidia in two ways (Barlow et al, 1977): (a) migration of screening pigments and (b) packing of the photoreceptor membrane (folds). Both lead to changes in the receptor's sensitivity to light (Chamberlain & Barlow, 1979; Barlow, 1985; Battelle, 1984; 2006).

3.5 Functional interpretations

The described structural and positional similarities of efferent visual cells in freshwater gastropods and in *Chelicerata* allow us to suggest that these cells carry out analogous functions. For *L. stagnalis* we suggested that such cells, receiving information from dermal photoreceptors, can be involved in the regulation of retinal light sensitivity (Tuchina et al, 2010), but published data on the presence of dermal photoreception in *P. corneus* do not exist.

There is still very little information on neurotransmitters, which can perform efferent modulations of the retinal cells in gastropods. There is no GABA-, octopamine- or

histamine-immunoreactivity in the eye or in the optic nerve of *Lymnaea* (Zhukov, 2007), and a similar lack of information was reported for *Bulla* by Michel et al (2000). However, as shown for *Aplysia*, the cerebral ganglion of this mollusc modulates the performance of the ocular pacemaker (Jacklet, 1984), and the putative transmitter here is serotonin, since it alone can phase-shift the rhythm (Corrent et al, 1978). Serotonin mimics ganglion attachment in case of experiments with the isolated eye (Nadakarukaren & Likey, 1986). Serotonin was also found to modulate responses of B-photoreceptors in *Hermissenda*; it significantly increases the amplitude and duration of the photoresponse evoked by light flashes with constant intensity (Crow & Bridge, 1985). The authors also suspected that serotonergic interneurons were activated by inputs from statocyst hair cells in *Hermissenda* and may then interact with B-photoreceptors. It was shown that exogenous serotonin can change the amplitude of the electroretinogram (ERG) and the light sensitivity of the isolated eye in *Lymnaea* (Zhukov et al, 2006) and that it is present in fibres of the optic nerve in this snail species.

In the optic nerve of *Bulla*, efferent fibres contain FMRF-amide and it was shown that this peptide suppresses the CAP activity, similar to what serotonin does in the eye of *Aplysia* (Jacklet et al, 1987). In the optic nerve of *Aplysia* no FMRF-amide-ergic fibres were revealed, and only exogenous FMRF-amide modulates the influence of light and serotonin on the circadian oscillator in the eye (Colwell et al, 1992).

We were interested whether serotonin or/and FMRF-amide are involved in the processing of the visual information in *Planorbarius*, and we found that both neurotransmitters are represented in the optic nerves, as well as in the eyes in this species. Serotonin- and FMRF-amide-ergic efferent fibres in the eye of *P. corneus* belong to neurons other than those, which send their processes in both optic nerves, and, thus, the chemical nature of these parietal and visceral neurons remains unclear. The presence of fibres, showing immunoreactivity to serotonin and FMRF-amide in the retina of *P. corneus*, allows us to suggest that these fibres play a modulatory role in adjusting retinal sensitivity levels to photic stimulation. Obviously, to confirm or reject this hypothesis requires further study.

Thus, we propose that the central neurons, which were identified in *P. corneus*, can control the adaptational state of the retina. It is hard to discuss their connections

with the circadian pacemaker since there is no information on the localization of the latter in *P. corneus*. But we found that besides the described central neurons, retinal cells can also be influenced by 5-HT and FMRF-amide-ergic-like fibres, which was not reported in previous studies.

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